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Prevalence of *Listeria monocytogenes* Subtypes in Bulk Milk of the Pacific Northwest

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ABSTRACT

The prevalence of *Listeria monocytogenes* in bulk milk from three Pacific Northwest states was assessed for 474 herds at three time points. For samples collected in November 2000 and June 2001, the *L. monocytogenes* prevalence levels were 4.9 and 7.0%, respectively. All isolates were subtyped by serotyping and by pulsed-field gel electrophoresis (PFGE). Forty-nine of the 55 isolates belonged to serogroup 1/2a, while 6 belonged to serogroup 4. Subtyping by PFGE revealed that isolates from 31 herds shared 10 patterns; there was a weak but significant association between PFGE subtype and geographical distance. Six herds were positive for *L. monocytogenes* at both time points. Of these six herds, four had indistinguishable PFGE patterns at both time points. Twenty-five of the 33 herds that were positive in June 2001 were sampled again in June 2002. *L. monocytogenes* was recovered from 17 of these 25 herds (68%), with the *Apa*I restriction enzyme digestion profiles (REDP) for 8 herds being identical to those of isolates recovered from these herds the previous year. The *Apa*I REDP for the bulk milk isolates were compared with those for isolates recovered from environmental and human samples that were collected by the Washington Department of Health ($n = 23$). Analysis of *Apa*I digestion profiles revealed that only two of the Washington Department of Health isolates had digestion profiles similar to those for isolates from bulk milk; however, further analysis with the use of a second enzyme (*Asc*I) was capable of discriminating between isolates from the two sources. Thus, we found no direct REDP matches between bulk milk and clinical isolates.

An estimated 76,000,000 people acquire foodborne illnesses annually in the United States; 325,000 of these people require hospitalization, and 5,000 die *(14).* Listeriosis, a zoonotic foodborne disease caused by *Listeria monocytogenes,* accounts for approximately 2,500 cases of illness and 500 deaths each year *(26).* Although listeriosis is relatively rare compared with other foodborne diseases, it frequently occurs with serious complications, including meningitis, septicemia, endocarditis, nonmeningitic central nervous system infection, febrile gastroenteritis, and abortion, in people with predisposing conditions. Neonates, elderly people, and immunocompromised people are particularly at risk. The United States currently maintains a zero tolerance policy for *L. monocytogenes* in food because of the severity of listeriosis *(8).*

Ready-to-eat meats, milk, and milk-related products have been implicated in several outbreaks of listeriosis *(29).* While thermotolerance studies have shown that pasteurization effectively destroys the bacterium *(9),* several outbreaks of listeriosis have been associated with pasteurized milk *(7, 8, 13),* probably because of contamination following pasteurization *(9, 16, 29).* Raw milk has been suggested to be a source of *L. monocytogenes* in the dairy processing environment *(16).* Between 2 and 16% of healthy cows excrete the organism in feces for months to years *(25, 31).* It has been hypothesized that fecal contamination and mastitis serve as sources of contamination of raw milk at the dairy farm *(15, 35).*

In order to better define the prevalence, distribution, and subtypes of *L. monocytogenes* in the Northwest, we sampled bulk milk from 474 herds in Washington, Oregon, and Idaho twice at a 7-month interval. To facilitate the examination of such a large number of samples, a modified enrichment method for isolating *L. monocytogenes* from milk was developed. Isolates were serotyped and further characterized by pulsed-field gel electrophoresis (PFGE) *(4, 23).*

MATERIALS AND METHODS

***Listeria* isolation from bulk milk.** Bulk milk was obtained from 474 herds in November 2000, June 2001, and June 2002 from a dairy cooperative and processed as follows. Milk was received in approximately 75-ml aliquots from the dairy cooperative and transported on ice to the laboratory. The milk was stored at 4°C and was processed within 3 weeks of arrival. The milk was shaken thoroughly, and a 50-ml aliquot was poured into a 50-ml conical tube. For herds from which multiple samples had been collected, equal aliquots of each sample were pooled. For example, if there were five samples for a herd, a 10-ml aliquot was taken from each sample and the five aliquots were pooled. The milk was clarified by centrifugation (3,200 × *g* for 30 min at 4°C). The top layer containing the fat was removed with a flamed spatula, and the supernatant was decanted. The resulting pellet was resuspended in 9 ml of University of Vermont medium (Remel, Lenexa, Kans.) by vortexing, and the suspension was incubated at 30°C for 24 h. After incubation, 50 μl of the University

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of Vermont medium was plated on modified Oxford agar (Remel) and incubated at 35°C for 48 h. After incubation, plates were inspected for colonies resembling *Listeria (28).* Three to five suspect colonies with typical *Listeria* morphological characteristics from each plate were characterized on the basis of CAMP test results, beta-hemolysis reaction, catalase reaction, Gram staining, and motility through semisolid media *(28).* A polymerase chain reaction (PCR) with *hlyA* was carried out to confirm the identity of each isolate exhibiting characteristics of *L. monocytogenes* as previously described *(2). L. monocytogenes* isolates were stored in brain heart infusion medium with 10% glycerol at −80°C.

Sensitivity of the isolation method. To estimate the sensitivity of our culturing technique, recently isolated *L. monocytogenes* strains of serotypes 1/2a ($n = 3$) and 4b ($n = 3$) were used to spike samples. Fifty milliliters of raw bulk milk was spiked with 0.5, 1, 2, or 10 CFU/ml and processed as described above. Experiments were performed in triplicate for each strain, resulting in a sample size of 18 for each inoculum level.

Comparison of culturing methods. To compare the recovery efficacies of the U.S. Department of Agriculture–Food Safety and Inspection Service (USDA-FSIS) method, the U.S. Food and Drug Administration *Bacteriological Analytical Manual* (FDA-BAM) method, and the centrifugation method described in the previous section of this paper, we tested 25 bulk milk samples in June 2002. The FDA-BAM method was performed as described by Hitchins *(21).* The USDA-FSIS protocol was adopted from Kornacki et al. *(24),* with 25 ml of milk being used instead of 25 g of food. For all three methods, up to five suspect colonies were picked from the selective agar medium and further characterized by the CAMP test and PCR as described above. Our centrifugation method differs from the USDA-FSIS and FDA-BAM methods with regard to the amount of enrichment media used (10 ml versus 225 ml) and the amount of time required for enrichment (24 h versus 48 h).

Serotyping. Denka Seiken *Listeria* antisera (Tokyo, Japan) were obtained from Accurate Scientific (Westbury, N.Y.). Serotyping was carried out according to the manufacturer's recommendations with the following modifications. Isolates were cultured in EB motility medium (3 g beef extract, 10 g peptone, 5 g NaCl, 4 g agar per 1 liter distilled water adjusted to pH 7.4) prior to H antigen determination. Growth from the outer edge of the motility medium was inoculated into 5 ml of Luria-Bertani medium and incubated at 30°C for 16 h. After the cells were harvested by centrifugation, the pellet was resuspended in 400 μl of 0.2% NaCl. One hundred microliters of the suspended antigen was combined with 40 μl of antiserum in a culture tube (6 by 50 mm). Agglutination reactions were recorded after incubation for 1 h at 51°C. For O antigen determination, 20 μl of cultured cells was used.

Genomic DNA preparation and PFGE conditions. Strain typing by the 30-h PFGE protocol was carried out as previously described *(17).* A standard isolate (F2365) was used to ensure uniform DNA preparation across experiments. The lambda size standard (Bio-Rad, Hercules, Calif.) was used as a molecular weight marker.

Analysis of PFGE restriction enzyme digestion profiles. BioNumerics (Applied Maths, Kortrijk, Belgium) was used for band detection and for the construction of dendrograms with the use of Dice binary coefficients and the unweighted pair group method with arithmetic averages (UPGMA). Visual inspection of bands was carried out according to BioNumerics's band assignment. A 1.5% tolerance was used for band matching. A standard isolate (F2365) was included in each gel to minimize gel-to-gel variability. Gels in which the standard isolate did not cluster within 95% similarity in the dendrogram were rejected from the analysis. Restriction enzyme digestion profiles (REDP) that were >95% similar according BioNumerics were visually inspected to ensure similarity. Isolates that had identical REDP for *Apa*I were analyzed with the use of a second enzyme (*Asc*I).

RESULTS AND DISCUSSION

Sensitivity of the isolation method. Previous *L. monocytogenes* isolation methods, such as cold enrichment *(20)* or selective enrichment *(21, 24),* require an extensive incubation period or a substantial amount of enrichment media. Our method for isolating *L. monocytogenes* from bulk milk was capable of recovering 1 CFU/ml from 14 (78%) of 18 spiked samples. Bias toward the recovery of a particular serotype was not observed. The strains used in this seeding experiment were propagated in nutrient media and thus were primed for replication. The sensitivity of this method in the detection of stressed or intracellular cells that may be present in natural contamination was not determined. Therefore, the sensitivity of this method for naturally contaminated samples may be weaker than indicated by spiking experiments.

Because of the large number of milk samples processed, milk samples were stored at 4°C for up to 3 weeks prior to testing. It is possible that this "cold enrichment" may have resulted in increased recovery of *L. monocytogenes.* Additionally, multiple milk samples were collected from many of the larger herds (with a range of one to nine samples and a mean of two samples), and all samples from a herd were pooled before testing. The presence of *L. monocytogenes* in a bulk milk sample may be due to environmental contamination of milk during collection, intramammary infections, or contaminated milking machinery. The effect of sample pooling is unclear. The pooling of samples may have reduced the sensitivity of the isolation procedure through the dilution of weakly contaminated samples. Alternatively, because a larger volume of milk was sampled, pooling may have increased the likelihood of detecting sporadic shedding.

To assess the effectiveness of different protocols in recovering *L. monocytogenes,* 25 bulk milk samples were tested in June 2002 by the USDA-FSIS, FDA-BAM, and centrifugation methods, which yielded recovery frequencies of 17 of 25, 8 of 25, and 11 of 25, respectively. Chi-square analysis revealed significant differences between the frequencies of *L. monocytogenes* recovery for the three methods ($\chi^2 = 6.73$, $P = 0.03$). Further cross-tabulation analysis revealed that there was a significant difference between the frequency of the recovery of *L. monocytogenes* for the USDA-FSIS method and that for the FDA-BAM method ($\chi^2 = 6.48$, $P = 0.01$), but not between the frequency for the USDA-FSIS or the FDA-BAM method and that for the centrifugation method ($\chi^2 = 2.92$, $P = 0.10$, and $\beta = 0.48$ when w [effect size] $= 0.24$; $\chi^2 = 0.76$, $P = 0.38$, and $\beta = 0.47$ when $w = 0.12$).

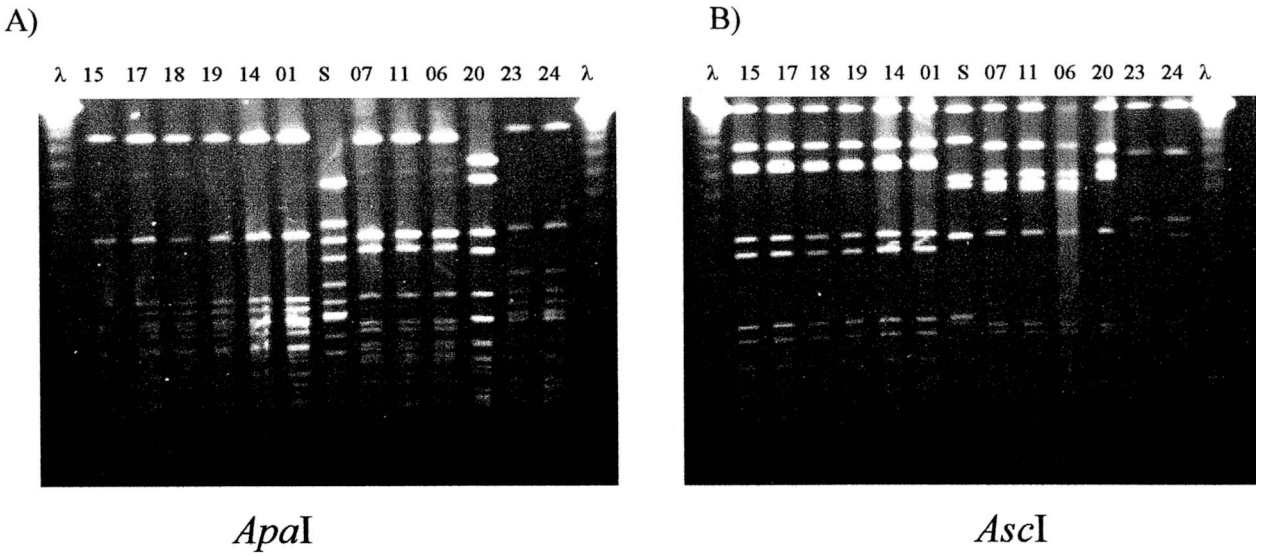

FIGURE 1. *PFGE REDP for isolates from the November 2000 sampling period after digestion with either* Apa*I (A) or* Asc*I (B). Each lane shows the REDP of a representative isolate from a single herd. Lane S, standard reference isolate (F2365); outermost lanes, lambda size standards.*

Prevalence and subtypes of *L. monocytogenes*. Of the bulk milk samples for 474 herds examined in November, 23 (4.9%) were positive for *L. monocytogenes*. All isolates were of serotype 1/2a. The prevalence of *L. monocytogenes* observed was consistent with previous reports from the Midwest United States, Trinidad, and Sweden *(22, 25, 33)*. Serotype 1 was the serotype most frequently isolated from environmental and milk samples in other studies *(11, 19, 33)*. The high prevalence of serotype 1/2a affirms that serology would have limited value in the epidemiology of *L. monocytogenes*.

The REDP obtained by PFGE with *Apa*I were identical for all isolates recovered within a herd (data not shown). Additionally, PFGE with *Apa*I and *Asc*I indicated that isolates from several herds shared indistinguishable macrorestriction patterns (Fig. 1A and 1B, respectively). Altogether, nine such patterns were obtained for 22 different bulk milk samples. One pattern was shared by isolates from seven different herds (herds 01, 14, 15, 17, 18, 19, and 22; Fig. 1A and 1B and Table 1).

Seven percent (33 of 474) of the bulk milk samples subsequently collected in June 2001 were positive for *L. monocytogenes*. As was the case for samples collected in November 2000, the majority of the isolates were of serotype 1/2a, although four isolates were of serotype 4b and two were of serotype 4c. Several isolates from different herds were found to have indistinguishable PFGE REDP when *Apa*I was used (*Apa*I REDP types B, F, G, H, I, N, O, R, and AK; Table 1). In addition, PFGE with *Apa*I could distinguish two strains in herd 07 (designated *Apa*I REDP types O and R in Fig. 2) with three band differences. One of the strains from herd 07 also shared a PFGE REDP with an isolate obtained from the same herd in November (*Apa*I REDP types O and R; Fig. 2 and Table 1). Both strains from herd 07 were of serotype 1/2a. All other herds had only one REDP. The difference between the proportion of positive herds in November (23/474) and that in June (33/474) was not statistically significant (Woolf's $\chi^2 = 1.88$, $P = 0.17$, $\beta = 0.02$ when $\alpha = 0.17$, $w = 0.2$) when the Mantel-Haenszel chi-square statistic (NCSS, Kaysville, Utah) was applied. This lack of significance, however, does not rule out seasonal variation.

L. monocytogenes was recovered from both the November 2000 and the June 2001 samples for six herds. All six strains were of serotype 1/2a. Isolates from four of the six herds had indistinguishable *Apa*I (Fig. 3) and *Asc*I (data not shown) REDP. For herd 08, there were six band differences between the November 2000 isolate and the June 2001 isolate. For herd 06, there were only two band differences between the November 2000 isolate and the June 2001 isolate (Fig. 3). Tenover *(32)* described isolates with two band differences as closely related and consistent with a single genetic event, such as an insertion or duplication of DNA.

To further study the subtypes of *L. monocytogenes* in bulk milk, 25 of the 33 herds that tested positive in June 2001 were sampled again in June 2002 (eight herds were not available for testing). *L. monocytogenes* was recovered from 11 of the 25 samples tested with our centrifugation method. *L. monocytogenes* was recovered from five additional samples by the USDA-FSIS method and from one additional sample by the FDA-BAM method. The herds from which *L. monocytogenes* was recovered by the USDA-FSIS and FDA-BAM methods are indicated in bold and italic type, respectively, in Table 1. As observed for the last two sampling periods, the predominant serotype was 1/2a, which was recovered from 14 of the 17 *L. monocytogenes*–positive bulk milk samples. *L. monocytogenes* isolates from the bulk milk samples of three of the herds were of serotype 4b. *Apa*I REDP were identical for all isolates recovered from the same bulk milk sample. *Apa*I REDP for isolates recovered from eight bulk milk samples were identical to those for isolates recovered from the same herds' bulk milk in June 2001. This finding supports earlier

TABLE 1. *Subtypes of* L. monocytogenes *isolated from bulk milk, human, and environmental samples*[a]

*Apa*I type	Herds yielding isolates of *Apa*I type for sampling date			WADOH isolates of *Apa*I type[b]
	November 2000	June 2001	June 2002[a]	
A		45	**08**	
B		27, 29	**29**	
C				841, 1163
D				842, 1164
E				9900101, 9900104
F		30, 37	30	
G		32, 36	36	
H	12, 16	43, 51	43	
I	04	04, 28		
J	06, 20, 21	48	**32**, 48	
K			37	
L		50	45, 50	
M	23, 24			
N		33, 35		
O	08, 10, 13	07, 10, 13	**46**	
P	01, 14, 15, 17, 18, 19, 22			
Q		41	27	
R	07, 11	07, 47	07	
S		44		
T		49		
U		46		
V				1160
W			**42**	
X				2140
Y				1167
Z				1161, 2172
AA				1329
AB	03			
AC			*44*	
AD		52		
AE		38		
AF		31		
AG		39		
AH		05		
AI	02			
AJ		06		
AK		08, 42		
AL		26	26, **33**	
AP				750
AQ				1155, 1445
AR				1159
AS				1165
AU				9900094
AV				1162
AW				1166
AX				2150
AY				1157
AZ				9900096

[a] Herds identified in bold and italic type cultured positive for *L. monocytogenes* by the USDA-FSIS and the FDA-BAM methods, respectively.

[b] WADOH, Washington Department of Health, Olympia, Wash.

reports that subtypes of *Listeria* can exist on a farm for an extended period *(25, 31, 33).*

For all three sampling times, four PFGE REDP were observed (*Apa*I REDP types J, O, H, and R; Table 1). Isolates from herd 07 obtained at all three sampling times had profile R. In addition, profile R was observed for isolates from bulk milk samples collected from three herds in November 2000 and June 2001.

The introduction of *L. monocytogenes* into the dairy environment was not investigated in this study. The finding that isolates from multiple bulk milk samples shared indistinguishable PFGE REDP was interesting. It is plausible

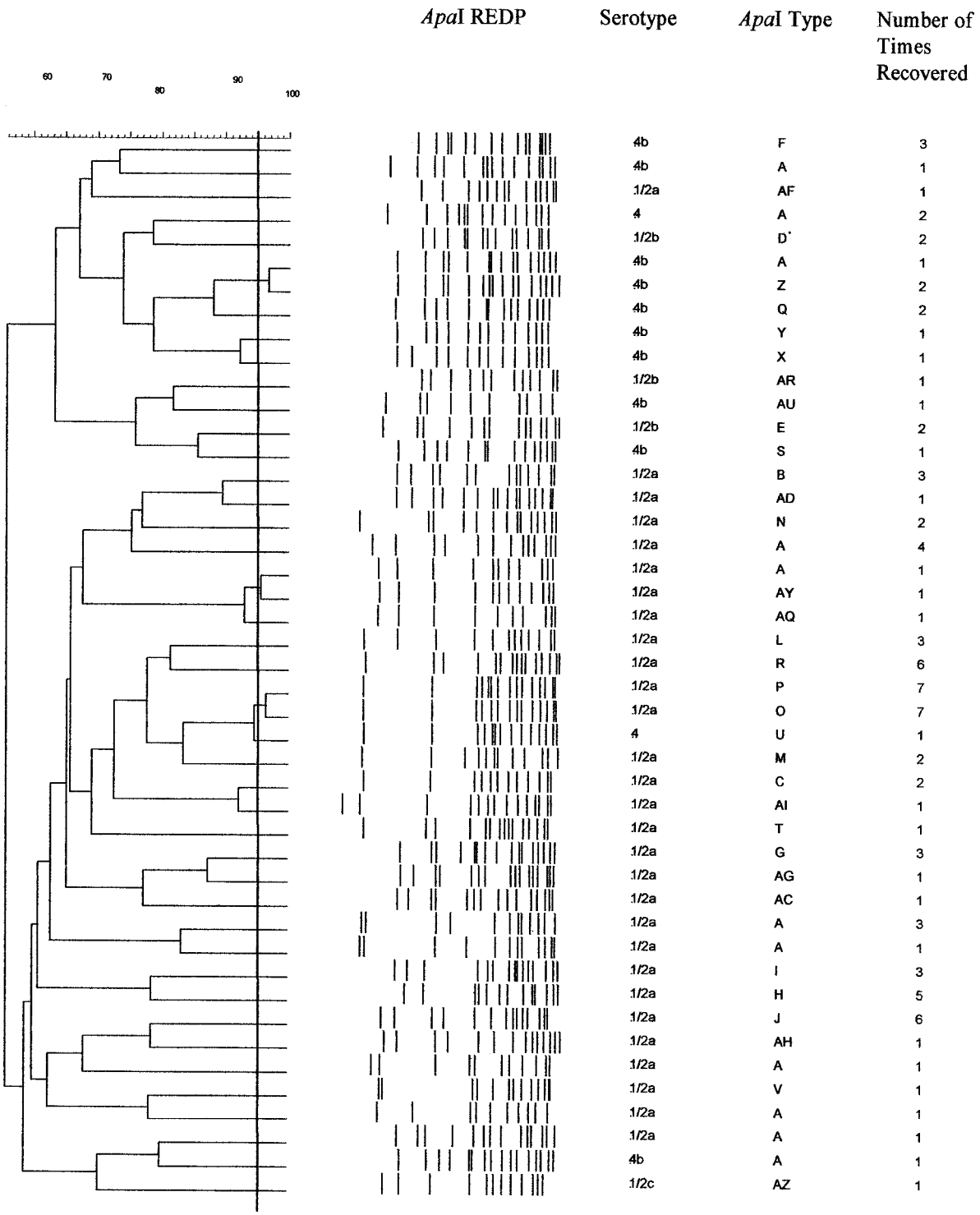

FIGURE 2. *UPGMA dendrogram of* Apa*I REDP for isolates from bulk milk and from the Washington Department of Health. The dendrogram was constructed from Dice coefficients with a 1.5% band-matching tolerance. The vertical line on the dendrogram indicates 95% similarity.*

that herds whose isolates have identical PFGE patterns are located in close proximity to each other. To test this hypothesis, the zip codes of the positive herds provided by the dairy cooperative were analyzed with a zip code map (http://zip.langenberg.com). The distances between zip code boundaries for herds with identical REDP ranged from 0 to 407 mi, with a mean of 158 mi. The association between REDP similarity and geographic distance was analyzed with PopTools (CSIRO, Australia). All pairwise comparisons of the geographic distance between two herds and the Dice similarity coefficient for the two associated isolates were calculated. The magnitude of the Mantel correlation was low ($r = -0.11$) but was significantly different from 0, as determined by random permutation ($P = 0.035$). There is a strong association between the feeding of improperly fermented silage to animals and animal listeriosis

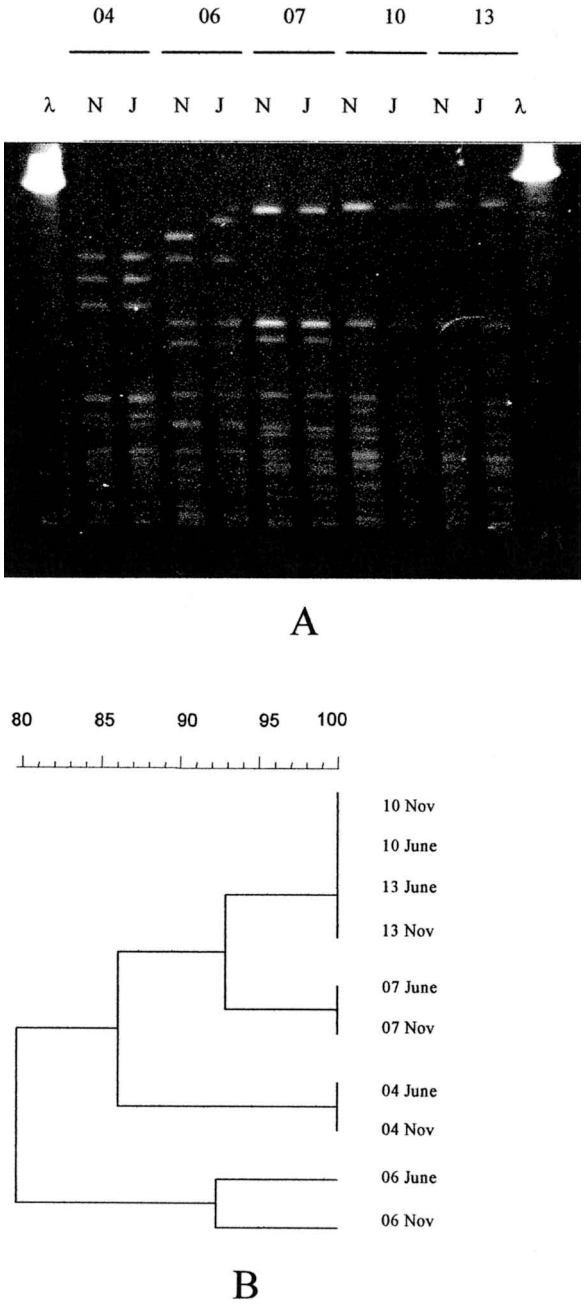

FIGURE 3. *Comparison of November 2000 and June 2001 PFGE REDP after* ApaI *digestion (A) and UPGMA dendrogram of* ApaI *REDP (B). Herd assignments are noted above lane designations (N, November isolates; J, June isolates). Outermost lanes, lambda size standards. The lowest band in the lambda size standard is 48.5 kb, with each successive band increasing by 48.5 kb. The dendrogram was constructed from Dice coefficients with a 1.5% band-matching tolerance.*

(10, 12, 30, 31, 34). Since most dairies produce their own silage, it is unlikely that silage of common origin is responsible for the dissemination of a subtype of *L. monocytogenes.* Commodities or feedstuffs of common origin may have resulted in the infiltration of these herds by a single subtype. Alternatively, the movement of livestock or wildlife may have been the source of contamination of bulk milk.

Because of the frequent isolation of strains with certain PFGE REDP from farm bulk milk, we analyzed the serotypes and REDP of 23 isolates recovered by the Washington Department of Health (WADOH) from 1999 to 2001. These isolates represented *L. monocytogenes* recovered from human patients and from environmental samples associated with the patients' illnesses. In contrast to bulk milk isolates, WADOH isolates demonstrated wider serotype diversity, comprising 7 serotype 4b isolates, 1 serotype 1/2c isolate, 5 serotype 1/2b isolates, and 10 serotype 1/2a isolates. Several isolates provided by the WADOH demonstrated *Apa*I REDP similar to those for isolates recovered from bulk milk in this study (WADOH REDP types 841 and 1163 and bulk milk REDP types 08, 10, 13, 07, and 46; Table 1 and Fig. 2). *Asc*I restriction enzyme analysis revealed a single-band difference (data not shown). In addition, genetic comparison of bulk milk isolates and WADOH isolates with the use of a mixed-genome DNA microarray also discriminated isolates collected from epidemiologically and geographically similar sources *(3).* Taken together, PFGE and microarray data suggest that there were no matches between isolates from bulk milk and isolates from human patients.

The two divisions that can be seen in Figure 2 were consistent with the correlation between serovar and genetic divisions identified by Brosch et al. *(5).* Other subtyping methods, such as those involving multilocus enzyme electrophoresis *(1),* ribotyping *(18),* mixed-genome microarrays *(3, 6),* and randomly amplified polymorphic DNA *(27),* have also classified strains into the two divisions seen in Figure 2. Although there is a strong precedence for two distinct divisions of *L. monocytogenes,* a biological significance (enhanced virulence, adaptive fitness) of the divisions has yet to be established.

In summary, the prevalence levels of *L. monocytogenes* in bulk milk samples obtained from herds in the Pacific Northwest in the winter of 2000 and in the summer of 2001 were 4.9 and 7.0%, respectively, with the preponderance of isolates belonging to serogroup 1/2a. The finding of certain PFGE REDPs being shared by several herds suggests limited diversity in the region or widespread distribution, possibly by contaminated feedstuffs, livestock, or wildlife. In addition, subtypes of *L. monocytogenes* can be isolated from a herd's bulk milk for at least 19 months. Although PFGE REDP types H, J, O, and R were recovered from bulk milk at all three sampling times, none of the subtypes isolated from bulk milk displayed PFGE and microarray subtypes *(3, 6)* identical to those for isolates recovered from human patients at about the same time and geographical location.

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